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Production of Alkaline Protease by a New *Aspergillus flavus* Isolate under Solid-Substrate Fermentation Conditions for Use as a Depilation Agent

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The production of alkaline protease by an *Aspergillus flavus* strain isolated in our laboratory by solid-substrate fermentation for use as a depilation agent and the influence of various factors on enzyme production are reported. The optimum conditions for maximum production were a growth temperature of 32°C, 63% substrate moisture, and a growth period of 48 h. Enrichment with corn steep liquor or Casitone increased productivity. Scaling-up experiments indicated that flask-scale results could be reproduced at 1 and 30 kg of substrate. The enzyme preparation exhibited maximum activity at both pH 7.5 and pH 9.5. The use of this enzyme as a depilation agent was confirmed by experiments in a tannery.

Tanners throughout the world are being increasingly affected by pollution and discharge control legislation. The pressures have been such that some tanneries have been forced to close down (3).

The most commonly practiced method for the depilation of hides and skins is the lime-sulfide process, which is now clearly recognized to be environmentally objectionable, accounting for 100% of the sulfide and over 80% of the suspended solids of tannery effluents (11). Sulfide is highly toxic and has an obnoxious odor. When sulfide is released into sewers, the accumulation of sulfide gases in the pipes causes corrosion and poisonous gases may also be released in the lower parts of the sewers.

Enzymatic depilation has been widely accepted as a sound alternative to the chemical process (12). However, the principal limiting factor for the large-scale rapid commercialization of enzymatic depilation has been the high cost of production of enzymes. Enzymes commercially available now are not economically comparable to the chemical process. Hence, any substantial reduction in the cost of production of enzymes will be a positive stimulus for the commercialization of enzymatic depilation.

Solid-substrate fermentation (SSF) was chosen for the present research because it has been reported that SSF with fungal strains results in much greater productivity than does submerged fermentation (4, 6). Economically, this type of fermentation possesses many advantages, including superior volumetric productivity, use of simpler machinery, use of an inexpensive substrate, simpler downstream processing, and lower energy requirements (2, 8). In addition, there is low wastewater output; consequently, there are fewer problems with waste treatment than are experienced with submerged fermentation (1).

The aim of this research was to devise a simple SSF process with a new *Aspergillus flavus* strain isolated in our laboratory and identified for its capacity to grow rapidly on a solid support and elaborate large quantities of extracellular proteases. Taking into account the economic profile of SSF, we report the production of alkaline protease by this *A. flavus* strain, including the optimization of various environmental factors, scaling-up trials, and results of trials in a tannery to confirm the use of the enzyme as a depilation agent.

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MATERIALS AND METHODS

Organism. The organism used in the present study is a new *A. flavus* strain, IMI 327634, isolated in our laboratory. The culture was routinely maintained in potato dextrose agar slants. Before each experiment, the organism was transferred to fresh slants and incubated at 28°C for 7 days. The spore suspension for inoculation was prepared by adding 10 ml of sterile distilled water to each slant and vigorously shaking the slant for 1 min.

Fermentation conditions. Commercial wheat bran (10 g) was thoroughly mixed with 15 ml of a salt solution containing, per liter, $NaNO_3$ (2 g), KH_2PO_4 (1 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), KCl (0.5 g), $FeSO_4 \cdot 7H_2O$ (trace), and $ZnSO_4 \cdot 7H_2O$ (trace), adjusted to pH 7.0 in 250-ml Erlenmeyer flasks, and sterilized at 15 lb/in^2 for 20 min. After cooling, the flasks were inoculated with 0.5 ml of the spore suspension. The contents were mixed thoroughly, incubated at 32°C for 2 days, and assayed for enzyme activity.

Optimization studies. Growth periods ranging from 12 to 168 h, growth temperatures ranging from 25 to 37°C, ages of the inoculum ranging from 2 to 10 days, different grades of wheat bran, different types of bran, different moisture levels (35 to 80%), effects of substrate cooking times (10 to 60 min), and effects of the addition of various carbohydrates and organic nitrogen sources were evaluated in relation to enzyme yields.

Enrichment of wheat bran with different carbohydrate sources was studied at the level of 2 g of carbohydrate per 10 g of wheat bran. The water-insoluble carbohydrates were thoroughly mixed with the wheat bran before moistening, and the water-soluble ones were dissolved in the salt solution and used for moistening the wheat bran. The heat-sensitive sugars (sucrose, lactose, and maltose) were separately sterilized and added to the sterile wheat bran medium before inoculation. Enrichment with different organic nitrogen sources was studied at the level of 1 g of organic nitrogen source per 10 g of wheat bran with a methodology similar to the one used for carbohydrates.

The experiments were conducted in triplicate, and the results are the averages of these three independent trials.

Extraction of the enzyme. At the end of the fermentation period, the entire quantity of moldy bran was homogenized with 200 ml of distilled water and filtered. The filtrate was used as the enzyme source.

For use as a depilation agent, the entire quantity of moldy bran at the end of the growth period was dried at 45°C in a hot-air-blowing oven and powdered.

Assay of enzyme activity. The activity of the enzyme was measured in terms of its action on casein. To 1 ml of casein solution (2.5%) were added 1.9 ml of glycine-sodium hydroxide buffer (0.1 M, pH 9.5) and 0.1 ml of enzyme, and the mixture was incubated at 42°C for 30 min. At the end of the incubation period, the reaction was arrested by the addition of 2 ml of 5% trichloroacetic acid. An enzyme blank was always included. The tubes were allowed to remain at room temperature for 30 min, and the solution was filtered through Whatman no. 1 filter paper. The optical densities of the trichloroacetic acid-soluble materials were read at 280 nm and compared with a tyrosine standard.

One unit of enzyme activity is defined as that amount of enzyme required to liberate 1 mg of tyrosine under assay conditions.

Tray-scale experiments. Experiments were conducted in enamel trays (1.5 ft by 1 ft by 2 in. [ca. 46 by 30 by 5 cm]) in which 500 g of wheat bran was mixed with 750 ml of salt solution, inoculated with the spore suspension, and incubated at 28°C.

Further scaling up in a pilot plant was done with 1 and 30 kg of substrate in a Koji room in which the temperature was maintained at 28°C and the humidity was kept at 90 to 95%. Perforated steel trays (3 ft by 1 ft by 2 in. [ca. 91 by 30 by 5 cm]) were used for this study. Wheat bran (1 kg) was spread in a thin layer (2 cm) after being mixed with 1.5 liters of salt solution. At the end of different incubation periods, samples were withdrawn and dried, and 1 g of dried enzyme was extracted with 50 ml of H_2O and assayed for activity.

pH profile and pH stability of the enzyme. Enzyme extract (100 μl) was preincubated with 1.9 ml of buffer (0.1 M citrate-phosphate, Tris-HCl, or glycine-NaOH) at pHs ranging from 3.0 to 11.0 and at 37°C for 2 h; 1 ml of 2.5% casein was added, and the mixture was incubated at 42°C for 30 min. To study the pH profile, we assayed the enzyme at different pHs.

Enzymatic depilation trials. Five pairs of goat skins were cut into halves. The left halves (controls) were painted with 10% lime and 2% sulfide (conventional depilation method). The right halves were painted with enzyme paste (1.0% enzyme, 7.0% kaolin, and 20% 10^{-5} M NaOH (pH 9.0) solution [wet weight]). The skins were piled flesh to flesh and incubated at 32 ± 1°C for 20 h. After incubation, hair from both sets was removed with a blunt knife. After depilation, cross sections of the pelts of the respective pairs were observed under a microscope. Skins were processed by standard methods, and the finished leathers were subjected to physical testing to evaluate strength properties. Both the control and the experimental leathers of each pair were cut perpendicularly and horizontally in identical regions for all of the tests.

RESULTS

Effects of growth temperature, substrate moisture content, and growth period. Temperature and moisture content of the substrate are the key factors which influence the outcome of an SSF system. Preliminary studies on growth and enzyme production at 25, 28, and 32°C indicated that although luxuriant growth occurred at all of these temperatures, productivity was low at 25°C and higher at 28 and 32°C.

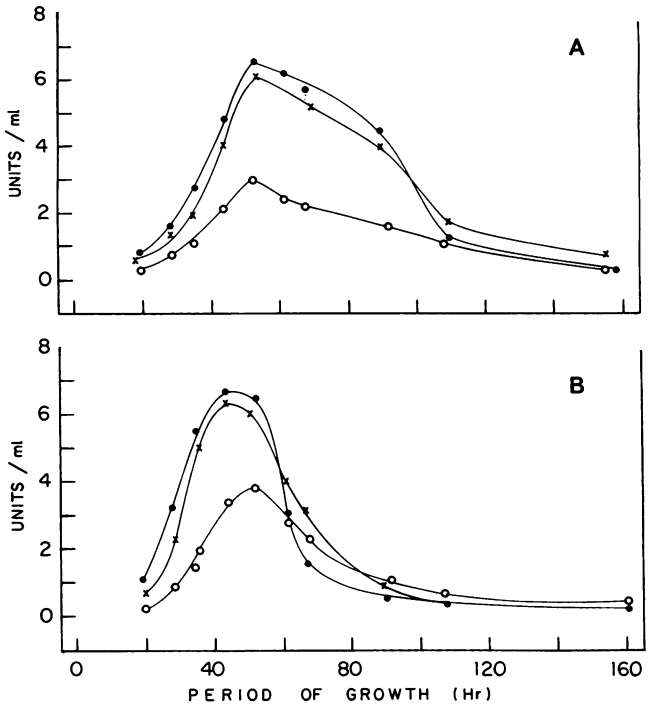

FIG. 1. Effect of growth period, moisture content of the substrate, and growth temperature on the production of alkaline protease by *A. flavus* IMI 327634. (A) Growth temperature of 28°C. (B) Growth temperature of 32°C. Symbols: ○, 52% moisture content; ●, 63% moisture content; ×, 70% moisture content.

A detailed correlation among the effects of moisture content, growth period, and growth temperature on enzyme production is shown in Fig. 1A and B. At a growth temperature of 28°C and at a substrate moisture content of 52%, the increase in enzyme activity was gradual, whereas at a 63 or 70% moisture content, there was a steep increase in enzyme activity from 40 h on, with the maximum being reached after 56 h of growth. Thereafter, there was a pronounced decrease in enzyme activity.

At a growth temperature of 32°C, a similar trend was observed. At a moisture content of 52%, there was a gradual increase in enzyme production. When the moisture content was 63 or 70%, there was a steep increase in enzyme activity from 36 h on, with a maximum (6.8 or 6.5 U/ml, respectively) being reached after 48 h. At this temperature, once the maximum was reached, there was a sharp decrease in enzyme activity; after 70 h, less than 50% of the maximum enzyme activity was retained. A comparison of enzyme production at 28 and 32°C indicated that the loss of enzyme activity was moderate at 28°C but pronounced at 32°C.

Effect of supplementation with organic nitrogen sources. The effect of the addition of various organic nitrogen sources (1 g/10 g of wheat bran) on protease synthesis is shown in Table 1. Corn steep liquor, Casitone, and yeast extract had a positive influence on productivity, resulting in 58, 42, and 33% increases over the control. Trader's protein (cottonseed-derived protein nutrient), peptone, malt extract, and

TABLE 1. Effect of different organic nitrogen sources on alkaline protease production by *A. flavus* IMI 327634[a]

Organic nitrogen source	Enzyme activity (U/ml)
Corn steep liquor	10.47 ± 1.76
Casitone	9.39 ± 1.01
Yeast extract	8.79 ± 0.86
Casein	6.20 ± 1.28
Albumin	6.04 ± 0.39
Soybean meal	6.11 ± 1.02
Trader's protein	5.68 ± 1.23
Peptone	5.46 ± 1.08
Malt extract	4.14 ± 1.35
Gelatin	2.98 ± 0.55
None (control)	6.60 ± 1.56

[a] The culture was grown at 28°C for 48 h. The moisture content of the wheat bran medium was 63%.

gelatin reduced enzyme synthesis considerably, and casein, soybean meal, and albumin had no effect.

Since casitone and corn steep liquor resulted in an increase in enzyme production, the effect of their concentrations was optimized (Table 2). In the case of corn steep liquor, the addition of 1 or 2 g resulted in enhanced enzyme synthesis, while higher concentrations repressed enzyme synthesis (Table 2). In the case of Casitone, only 1 g resulted in increased enzyme synthesis, while higher concentrations reduced enzyme synthesis considerably (Table 2).

Effect of the addition of carbohydrates. The influence of the addition of various carbohydrates (2 g/10 g of wheat bran) is shown in Table 3. All sugars tested, except for lactose, severely repressed enzyme synthesis.

Effect of the nature of the substrate. The effect of different types of bran (wheat bran, rice bran, and bran from different pulses) on enzyme production was studied. Of all the brans tested, only wheat bran could elaborate enzyme activity. All of the other brans supported luxuriant growth but no enzyme activity. Coarse and fine wheat brans of mesh sizes 0.3 to 0.4 cm and 0.05 to 0.1 cm, respectively, were evaluated for their effect on enzyme yields. Enzyme activity was 80% in the presence of the fine variety when the yield in the coarse variety was considered 100%.

Other factors, such as age of the inoculum and sterilization time, were also evaluated. A 5- to 7-day-old culture showed maximum enzyme production. Extended sterilization (45 min at 15 lb/in^2) resulted in maximum enzyme production.

Scaling-up studies. Increasing the size of the substrate from 10 g in a 250-ml flask to 500 g in trays indicated that flask-scale results could be reproduced. Further scaling up to 30 kg in a Koji room resulted in a higher yield (about 30% over the flask-scale yield) in a shorter span of time (35 h).

TABLE 2. Effect of various concentrations of corn steep liquor and Casitone on alkaline protease production by *A. flavus* IMI 327634[a]

Level of enrichment (g/10 g)	Enzyme activity (U/ml) with:	
	Corn steep liquor	Casitone
1	10.68 ± 1.85	9.46 ± 0.82
2	10.68 ± 1.69	5.00 ± 2.33
3	6.56 ± 1.43	2.48 ± 0.85
4	0.20 ± 0.04	1.43 ± 0.29
5	0.10 ± 0.05	0.97 ± 0.19

[a] The culture was grown at 28°C for 48 h. The moisture content of the wheat bran medium was 63%.

TABLE 3. Effect of various carbohydrates on alkaline protease production by *A. flavus* IMI 327634[a]

Carbohydrate	Enzyme activity (U/ml)
None (control)	6.54 ± 2.0
Lactose	7.03 ± 0.95
Sucrose	3.74 ± 1.11
Starch	3.72 ± 1.2
Fructose	3.47 ± 0.45
Dextrin	3.33 ± 0.62
Maltose	3.19 ± 0.72

[a] The culture was grown at 28°C for 48 h. The moisture content of the wheat bran medium was 63%.

Figure 2 shows the pH profile of the enzyme, with maxima at pH 7.5 and pH 9.25. pH stability studies showed that the enzyme was unstable as the alkalinity increased.

Results of trials carried out in the experimental tannery to evaluate the use of this enzyme as a depilation agent indicated that enzyme treatment did not have any detrimental effect on the final leathers obtained. Table 4 shows the physical evaluation data for the finished leathers from both groups. The tensile strength, stitch tear strength, and tongue tear strength were better in the experimental set. Bursting strength and elongation at the break point were also better in the experimental set. Microscopic evaluation of the depilated pelts also indicated that fiber structures were well opened up and that there were no visible fat glands in the experimental pelts.

DISCUSSION

It is known that the water content of a medium has a profound influence on the growth of and production of products by microorganisms. In an SSF the water content of

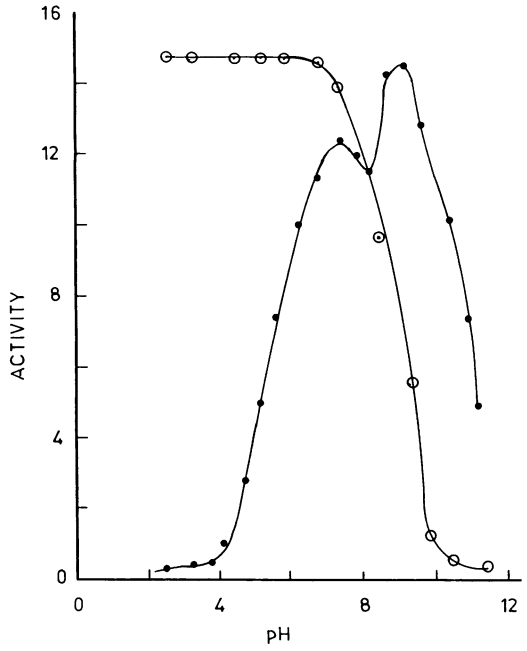

FIG. 2. pH profile (●) and pH stability (○) of *A. flavus* alkaline protease.

TABLE 4. Physical evaluation data

Sample and cut of leather	Tensile strength (kg/cm^2)	Elongation at break (%)	Stitch tear strength (kg/cm^2)	Tongue tear strength (kg/cm)	Bursting strength (kg/cm)
Control					
Perpendicular	204.06 ± 3.22	37.5 ± 1.5	65.34 ± 0.13	39.77 ± 1.28	300.00 ± 2.45
Parallel	218.09 ± 2.38	50.0 ± 1.5	68.18 ± 1.29	42.61 ± 0.97	
Experimental					
Perpendicular	214.26 ± 5.26	62.5 ± 1.39	74.10 ± 5.64	48.30 ± 1.71	325.00 ± 3.5
Parallel	228.17 ± 6.32	62.5 ± 3.0	85.23 ± 7.64	53.98 ± 0.69	

the substrate (5, 10) is greatly influenced by the absorbing capacity and capillary forces of the substrate, the growth temperature, the amount of metabolic heat generated, the quantity of moisture evolved, and the growth requirements of the organism. Lindenfelser and Ciegler (7) noted for ochratoxin A production by *A. ochraceous* in an SSF that the initial wheat bran moisture content was the most critical of all the fermentation conditions studied. Wang et al. (14) reported that 50 to 63% was sufficient for acid protease production by three strains studied by them under SSF conditions. In the case of alkaline protease production by *A. flavus*, a moisture content of 52 to 63% favored maximum production. A temperature of 28 to 32°C was essential for both efficient growth and maximum enzyme secretion.

The rapid decline in enzyme activity after the maximum was reached may have been due to the alkaline shift of the medium as the period of growth was extended (Fig. 3). Our studies on the pH stability of the enzyme (Fig. 2) indicated that the enzyme was increasingly unstable as the pH increased above 8.0. Another factor contributing to the loss of enzyme activity could have been that the depletion of the nitrogen sources present in the wheat bran may have been triggering the utilization of the enzyme as a protein nitrogen source by the organism. A similar observation has been reported in the case of a *Cephalosporium* sp. (13).

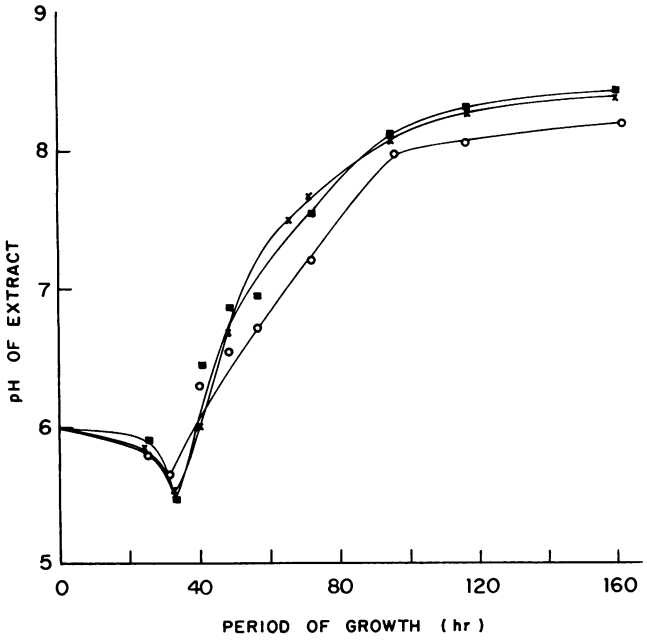

FIG. 3. Variations in the pH of the moldy bran extract with respect to period of growth at 28°C and at different moisture levels. Symbols: ○, 52% moisture; ■, 63% moisture; ×, 70% moisture.

Earlier studies in liquid cultures on the production of extracellular proteases by *A. flavus* indicated that this is an inducible system in this organism, requiring the presence of specific organic protein sources for synthesis (9). All protein sources do not serve as inducers. Our studies on protease synthesis by *A. flavus* in an SSF indicate that the proteins associated with the starch in wheat bran serve as inducers. Hence, wheat bran alone is sufficient to elaborate enzyme activity. Protein sources such as yeast extract, corn steep liquor, and Casitone can also serve as specific inducers, thereby exerting a cumulative positive influence on enzyme production. Proteins having a negative influence on enzyme production may inhibit enzyme synthesis because of feedback inhibition.

The coarser variety of bran appeared to be a better substrate, probably because it does not form a compact mass and permits better air circulation, heat dissipation, and penetration by mycelia. Also, it is a better prospect economically since it is cheaper than the finer variety of bran. Extended sterilization allows this coarse wheat bran to be used in a form such that the nutrients present in it are easily available to the organism. The repression of enzyme synthesis by most of the sugars tested is attributed to the catabolite repression effect, in which the presence of easily assimilable carbon sources restricts enzyme synthesis.

It is well recognized that the moisture content of the substrate plays a crucial role in SSF systems. The initial moisture content varies during fermentation because of evaporation, metabolic activity, and generation of metabolic heat and becomes particularly important when SSF is carried out on a large scale. This loss is compensated for by the maintenance of high humidity (90 to 95%) in the chamber in which the fermentation is carried out. Scaling-up experiments in a Koji room in which the humidity was maintained at 90 to 95% resulted in a higher productivity than that achieved in flask- and tray-scale experiments carried out in the laboratory.

SSF is akin to the natural habitat of fungi. Studies have revealed that productivity is sometimes better under such conditions than under submerged fermentation conditions (14).

Enzymes so far reported for this purpose have been produced by submerged fermentation at a prohibitively expensive price, which has made tanners reject enzymatic depilation. We have attempted to combine the economic advantages of an SSF system with the capacity of the new *A. flavus* strain isolated in our laboratory to grow very rapidly and produce prolific amounts of extracellular proteases such

that the final enzyme product is available at a commercial price attractive to tanners. Extensive trials carried out in an experimental tannery have confirmed the successful use of this enzyme as a depilation agent. The use of this enzymatic depilation process completely eliminates the use of sulfide, an environmentally objectionable pollutant.

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REFERENCES

1. **Barreto de Menezes, T. J., T. De J. G. Salva, V. L. Baldini, R. S. Papini, and A. M. Sales.** 1989. Protein enrichment of citrus wastes by solid substrate fermentation. Proc. Biochem. **24**:167–171.
2. **Cannel, E., and M. Moo-Young.** 1980. Solid state fermentation systems. Process Biochem. **6**:2–7.
3. **Davighi, D.** 1988. Keeping the environment clean. World Leather **1**:29–32.
4. **Ghildyal, W. P., B. K. Lonsane, K. R. Sreekantiah, and V. Sreenivasamurthy.** 1985. Economics of submerged and solid state fermentations for the production of amyloglucosidases. J. Food Sci. Technol. **22**:171–176.
5. **Grevais, P., J. M. Belin, W. Grajek, and M. Sarrette.** 1988. Influence of water activity on aroma production by *Trichoderma viride* TS growing on a solid substrate. J. Ferment. Technol. **66**:403–407.
6. **Hesseltine, C. W.** 1972. Solid state fermentations. Biotechnol. Bioeng. **14**:517–532.
7. **Lindenfelser, L. A., and A. Ciegler.** 1975. Solid substrate fermentation: consistent improved yields. Biotechnol. Bioeng. **30**:267–271.
8. **Lonsane, B. K., N. P. Ghildyal, S. Budiatman, and S. V. Ramakrishna.** 1985. Engineering aspects of solid state fermentation. Enzyme Microb. Technol. **1**:258–265.
9. **Malathi, S., and S. C. Dhar.** 1987. Production of extracellular proteases by an *Aspergillus flavus* isolate and its application in the depilation of skins. Leather Sci. **34**:67–76.
10. **Narahara, H., Y. Koyama, T. Yoshida, S. Pichangkura, R. Ueda, and H. Taguchi.** 1982. Growth and enzyme production in solid state culture of *Aspergillus oryzae*. J. Ferment. Technol. **60**: 311–319.
11. **Pepper, K. W., and K. G. E. Wyatt.** 1989. Enzyme unhairing of heavy hides. J. Indian Leather Technol. Assoc. **36**:214–233.
12. **Taylor, M. M., D. G. Bailey, and S. H. Feairheller.** 1987. A review of the uses of enzymes in the tannery. J. Am. Leather Chem. Assoc. **81**:85–102.
13. **Tsuchiya, K., and T. Kimura.** 1984. Decrease in protease activity by the addition of glucose to the culture of *Cephalosporium* sp. J. Ferment. Technol. **62**:35–39.
14. **Wang, H. L., J. B. Vespa, and C. W. Hesseltine.** 1974. Acid protease production by fungi used in soybean food fermentation. Appl. Microbiol. **27**:906–911.